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Down-regulation of human telomerase reverse transcriptase through specific activation of RNAi pathway quickly results in cancer cell growth impairment

Paolo Gandellini [a,1], *Marco Folini* [a,1], *Roberto Bandiera* [a], *Michelandrea De Cesare* [a], *Mara Binda* [a], *Silvio Veronese* [b], *Maria Grazia Daidone* [a], *Franco Zunino* [a], *Nadia Zaffaroni* [a,*]

[a] *Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy*
[b] *Department of Pathology, Azienda Ospedaliera Niguarda, Milano, Italy*

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ABSTRACT

Targeting of human telomerase reverse transcriptase (hTERT) by different small interfering RNAs (siRNAs) resulted in a variable degree of telomerase activity inhibition in PC-3 and DU145 prostate cancer cells. In addition, transfection with siRNA5 and siRNA41, which caused high levels (~80 and ~55%, respectively) of enzyme activity inhibition in both cell lines, led to a marked reduction of hTERT mRNA and protein expression and a significant inhibition of cell proliferation within a few days, without concomitant telomere shortening or telomeric 3′ overhang impairment. Such an antiproliferative effect was not ascribable to the activation of non-specific responses, since siRNA5 and siRNA41 did not induce the expression of 2′–5′ oligoadenylate synthetase-1 and were able to cause a significant growth impairment also in HCT 116 colon cancer cells, which have a defective interferon pathway. Cell growth inhibition was indeed associated with hTERT down-regulation, as it was almost completely rescued in siRNA-treated HCT 116 cells co-transfected with an hTERT-expressing vector. Moreover, siRNA5 and siRNA41 failed to affect the proliferation of hTERT-negative U2-OS osteosarcoma cells. Interestingly, transfection with siRNA5 significantly reduced the tumorigenic and growth potential of PC-3 cells when xenotransplanted into nude mice. Such data suggest siRNA-mediated hTERT down-regulation as an efficient strategy to impair prostate cancer cell growth.

1. Introduction

Telomerase is a ribonucleoprotein complex whose principal role is the maintenance of telomeres, the heterochromatic structures made of G-rich repeated sequences that cap and protect the ends of chromosomes [1]. Human telomerase is composed of two main subunits: the human telomerase RNA (hTR), which harbors an 11 nucleotide long sequence acting as a template for the synthesis of telomeric repeats, and the human telomerase reverse transcriptase (hTERT), which is the core catalytic subunit of the enzyme [2]. When correctly assembled, telomerase contributes to lengthen chromosome

* *Corresponding author*. Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian, 1-20133 Milano, Italy. Tel.: +39 02 2390 3260; fax: +39 02 2390 3052.
E-mail address: nadia.zaffaroni@istitutotumori.mi.it (N. Zaffaroni).

[1] The authors contributed equally to this work.

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termini, thus counteracting telomere erosion that occurs at each round of cell division [3]. Recent findings have contributed to uncover unexpected extratelomeric functions of telomerase. Specifically, it has been demonstrated that hTERT preserves the integrity of the ends of chromosomes by contributing to T-loop formation and by protecting telomeres in unclosed loops [4,5], thus preventing them from resembling DNA double-strand breaks [4,5]. In addition, novel functions of telomerase, that might have a potentially important role in tumor cells, are related to the ability of hTERT to cross-link telomeres and enhance genomic stability and DNA repair [6], as well as to maintain tumor cell survival and proliferation via enzymatic activity-independent intermolecular interactions involving p53 and poly-(ADP-ribose) polymerase [7]. Altogether such findings have provided hTERT with a pro-survival role, which is independent of its telomere-elongating activity and could be of great impact in the tumorigenesis process.

With the exception of germ and stem cells, and cells belonging to specific compartments of highly proliferative tissues (e.g. basal layer of epidermis) [8], telomerase is generally repressed in normal tissues, whereas it is reactivated in a vast majority of human tumors [9]. Such a tumor-specific expression has contributed to identify telomerase as a promising target for the development of new anticancer therapies [10–12]. Thus far, several strategies relying on antisense oligonucleotides, ribozymes and PNAs targeting either hTR or hTERT component have been developed to interfere with telomerase expression in several tumor experimental models [13]. Recently, a post-transcriptional gene silencing pathway mediated by double-stranded RNA (dsRNA) and referred as to RNA interference (RNAi) has been described [14]. RNAi is a natural mechanism of defence, which protects cells against exogenous dsRNA, such as viral or deriving from transposones. When a dsRNA enters the cytoplasm, RNAse III Dicer can process it to produce several small interfering RNAs (siRNAs), 21–23 nucleotide long RNA molecules with 2 nucleotide long 3′ overhangs [15]. Small interfering RNAs may get incorporated into the RNA induced silencing complex (RISC), which identifies and silences complementary RNAs generally through a cleavage mechanism [15]. In the last few years, it has been demonstrated that siRNAs represent an efficient tool to modulate the expression of a large number of cancer-related genes [16]. In the present study, we investigated (i) the possibility to efficiently down-regulate hTERT expression through the use of chemically synthesized siRNAs targeting different consensus sequences within hTERT mRNA, and (ii) the consequences of the inhibition of hTERT expression and telomerase activity on the proliferative potential and tumorigenicity of human prostate cancer cells.

2. Materials and methods

2.1. Cell lines

Four telomerase-positive cell lines (HeLa S3 cervix carcinoma, PC-3 and DU145 androgen-independent prostate adenocarcinoma and HCT 116 colorectal carcinoma) and a telomerase-negative cell line (U2-OS osteosarcoma) were used. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in logarithmic growth phase at 37 °C, in 5% CO_2 humidified atmosphere, in their own culture medium, RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 0.1% gentamycin (HeLa S3, PC-3, DU145 and HCT 116) and Mc Coy's supplemented with 10% FCS (U2-OS).

2.2. Design and synthesis of small interfering RNAs

Fourteen different siRNAs targeting specific consensus sequences (5′-AA(N_{19})-3′, where N is any nucleotide) within the open reading frame of hTERT mRNA (GeneBank accession no. AF015950) were designed using siRNA target finder tool (http://www.ambion.com/techlib/misc/siRNA_finder). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) for selected siRNA sequences was performed to exclude any alignment with other sequences in the human genome. Small interfering RNAs were manufactured by MWG-Biotech (Ebersberg, Germany) as pre-formed and purified duplexes, made of 19 bp long RNA oligonucleotides with two extra-thymidine bases forming a 3′ overhang on both strands. Each siRNA was resuspended in sterile RNase-free water, diluted to the appropriate working solution (20 μM) and stored at −20 °C until use. A control siRNA, made of a scrambled sequence with no significant homology to any known human mRNA, was used throughout the study [17]. In addition, a siRNA (siRNA GFP) targeting the consensus sequence 5′-AAGGACGACGGCAACUACAAG-3′ within the enhanced green fluorescent protein (EGFP) mRNA (GeneBank accession no. U55762) encoded by the pEGFP-N1 reporter vector (BD Biosciences Clontech, Palo Alto, CA) was used.

2.3. Cell culture and transfection procedures

For transfection experiments, a given amount of each siRNA was mixed to Lipofectamine-2000™ reagent (Invitrogen, Carlsbad, CA) for 20 min at room temperature according to the manufacturer's instructions. The mixtures were then applied to cells, seeded out in media without antibiotics 24 h before transfection, in an appropriate volume of Opti-MEM I medium (Invitrogen), so that the final concentration of siRNA was 125 nM. After a 4 h incubation at 37 °C, cells were washed with phosphate buffered saline and culture medium supplemented with serum was added. Cells were then cultured for different intervals of time, harvested and subsequently analyzed. Cells exposed to only Lipofectamine-2000™ were referred to as mock control along the study. In co-transfection experiments, 1 μg of pEGFP-N1 vector or of an hTERT-expressing vector (pCI-neo-hTERT, kindly provided by Prof. R.A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MT) was added to the siRNA/Lipofectamine-2000™ mixtures and cells transfected as described above.

2.4. Cellular uptake of siRNAs

To assess cellular uptake, siRNAs were labeled with carboxyfluorescein (FAM) using Silencer™ siRNA Labeling Kit (Ambion, Austin, TX), according to the manufacturer's instructions. Cells

were transfected as described above with 125 nM FAM-conjugated siRNAs. At the end of transfection, cells were washed twice with phosphate buffered saline and subsequently analyzed under a fluorescence microscope (Eclipse TS100, Nikon, Tokyio, Japan). Images were acquired using a DC290 digital camera (Eastman Kodak Company, Rochester, NY).

2.5. Telomeric repeat amplification protocol assay (TRAP)

Telomerase activity from 0.05 μg of cell protein extracts was analyzed by the PCR-based TRAP assay, using TRAPeze Kit (Intergen, Oxford, UK). Each reaction product was amplified in the presence of a 36-bp internal control (ITAS) for the TRAP assay, and each sample extract was also incubated at 85 °C for 10 min to test its heat sensitivity. A TSR8 quantitation standard (which serves as a standard to estimate the amount of product extended by telomerase in a given extract) was included for each set of TRAP assays. Quantitative analysis was performed with the Image-QuanT software (Molecular Dynamics, Sunnyvale, CA), which allowed densitometric evaluation of the digitized image. Telomerase activity was quantified by measuring the signal of telomerase ladder bands, and the relative telomerase activity was calculated as the ratio to the internal standard using the following formula:

$$\text{relative telomerase activity}: \left[\frac{(X - X_0)}{C}\right] \times \left[\frac{R - R_0}{Cr}\right]^{-1},$$

where X is the sample, X_0 the heat-inactivated sample, C the internal control of the sample, Cr the internal control of TSR8, R the TSR8 quantitation control and R_0 is the negative control.

2.6. RT-PCR and TaqMan® real time RT-PCR

Total RNA was isolated from siRNA-transfected cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and reverse-transcribed using the GeneAmp RT-PCR Core Kit (Applied Biosystems, Foster City, CA). hTERT mRNA expression levels were assessed by performing 33 cycles of PCR (95 °C, 30 s; 65 °C, 45 s; 72 °C, 30 s; forward primer: 5′-GCCTGAGCTGTACTTTGTCAA-3′; reverse primer: 5′-GCAAACAGCTTGTTCTCCATGTC-3′) in the presence of 1.5 μCi of [α-^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol, GE Healthcare Europe GmbH, Cologno Monzese, Italy). PCR products were run on polyacrylamide gel and detected by autoradiography. 2′–5′ Oligoadenylate synthetase-1 (OAS-1) mRNA expression levels were assessed by performing 35 cycles of PCR (95 °C, 30 s; 60 °C, 45 s; 72 °C, 20 s; forward primer: 5′-AGGTGGTAAAGGGTG GCTCC-3′; reverse primer: 5′-ACAAC-CAGGTCAGCGTCAGAT-3′). The amplicons were visualized by agarose gel stained with ethidium bromide. β-Actin was amplified (forward primer, 5′-GAATTCAAAACTGGAACGGT-GAAGG-3′; reverse primer, 5′-AAGCTTATCAAAGTCCTCGGC-CACA-3′) and used as a standard for each PCR analysis. To quantify the extent of siRNA-mediated hTERT down-regulation, real-time RT-PCR was performed. Reverse transcription and amplification were performed on total RNA according to established TaqMan® PCR assay protocol. Primers and probe for hTERT mRNA (TaqMan® Gene Expression Assay part no. 4367301B) and RNaseP (TaqMan® RNaseP control reagents part no. 4316844) were obtained from Applied Biosystems. Relative quantification of hTERT mRNA expression levels was accomplished by the $2^{-\Delta\Delta C_T}$ method, as described in Ref. [18]. RNaseP was used as normaliser and cells exposed to control siRNA as calibrator.

2.7. Cell growth inhibition assay

Cells in logarithmic growth phase were seeded at 0.2×10^6 (PC-3) and 0.15×10^6 (DU145) in each well of six-well plates and transfected with 125 nM siRNAs as described above. At the end of treatment cells were washed with phosphate buffered saline and incubated at 37 °C in a 5% CO_2 humidified atmosphere for additional 1–6 days. Adherent cells were then trypsinized and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in siRNA-treated samples.

2.8. Western immunoblotting

Total cellular lysates were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% skimmed milk and incubated overnight with primary antibody specific for hTERT (Rockland, Gilbertsville, PA). Filters were then probed with a horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare Europe) for 1 h. Bound antibodies were detected using enhanced chemoluminescence Western blotting detection system (GE Healthcare Europe). An anti-β-actin monoclonal antibody (Abcam, Cambridge, UK) was used on each blot to ensure equal loading of protein on the gel. Results were quantified by densitometric analysis.

2.9. Evaluation of DNA content

Samples made of 1×10^6 cells were fixed in 70% (v/v) ethanol and subsequently stained with a solution containing 50 μg/ml propidium iodide, 10 mg/ml RNase, and 0.05% Nonidet P-40 for 30 min at room temperature and analyzed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). A flow-cytometric sub-G_1 peak was detected on DNA plots using the CellQuest software according to the Modfit model (Becton Dickinson).

2.10. Southern blot analysis of telomere length

Total DNA was isolated from siRNA-treated cells using DNAzol (Invitrogen). Twenty micrograms of DNA were digested with Hinf I, electrophoresed on 0.8% agarose gel and transferred to a nylon membrane. The membrane was then rapidly washed, cross-linked and hybridized with a ^{32}P-labeled telomeric probe $(TTAGGG)_4$. After hybridization the membrane was exposed to an autoradiography film. Images were acquired by a ScanJET IIcx/T scanner (Hewlett Packard) and analyzed by Image-QuanT software (Molecular Dynamics). The mean terminal restriction fragment (TRF) length was then calculated as previously reported [19].

2.11. *In solution hybridization assay*

Aliquots of 5 μg genomic DNA, undigested and digested with Exo I were hybridized for 18 h at 37 °C with 0.5 pmol of the ^{32}P-labeled probe $(CCCTAA)_4$, in a hybridization buffer containing 10 mM Tris–HCl pH 8.0, 12 mM $MgCl_2$, 1 mM DTT, for a final volume of 20 μl. Reactions were stopped by the addition of 4 μl of loading buffer 6× and samples were size fractionated on 1% agarose gel. The gel was dried and exposed to an autoradiography film. The relative hybridization signal was normalized to that obtained from the same gel previously stained with ethidium bromide.

2.12. *In vivo studies*

Experiments were carried out using 7–8-week-old male athymic Swiss nude mice (Charles Rivers, Calco, Italy). The mice were kept in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy) according to the United Kingdom Coordinating Committee on Cancer Research Guidelines [20]. To assess tumorigenicity of PC-3 cells, untreated cells and cells collected immediately after a 4-h transfection with control siRNA or hTERT-specific siRNAs were injected subcutaneously into both flanks of the mice (10^7 cells/flank). Animals (five mice for each group) were inspected daily to establish tumor take, time of tumor appearance and tumor volume. Tumor growth was followed by measuring tumor diameters with a Vernier caliper. Tumor volume was calculated according to the formula: TV (mm^3) = $d^2 \times D/2$ where d and D are the shortest and longest diameters, respectively. For ethical reasons, mice bearing established tumors were sacrificed when the TV was around 1000 mm^3.

2.13. *Statistical analysis*

Student's *t*-test was used to analyze the differences between control and hTERT-specific siRNA-transfected cells in terms of hTERT mRNA expression levels and cell growth as well as for comparison of the volume of tumors growing in nude mice. Fisher's exact text was used to compare tumor take in the different animal groups. All tests were two-sided. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. *siRNAs are efficiently internalized and inhibit telomerase activity in prostate cancer cells*

We assessed the ability of fourteen chemically synthesized siRNAs (Fig. 1a) to interfere with telomerase function in PC-3 and DU145 androgen-independent prostate cancer cell lines. Carboxyfluorescein (FAM) labeling was instrumental to detect the uptake and intracellular distribution of siRNAs by fluorescence microscopy. After a 4-h transfection in the presence of Lipofectamine-2000™, FAM-labeled siRNAs were present and homogeneously distributed in the cytoplasm of PC-3 cells (Fig. 1b). A similar pattern of intracellular siRNA distribution was observed in DU145 cells (data not shown). Once verified the uptake and intracellular distribution of siRNAs, we evaluated their ability to inhibit telomerase activity in the TRAP assay, carried out in cells collected 2 days after a 4-h transfection with 125 nM of each hTERT-specific siRNA. A preliminary screening was performed in the HeLa S3 cervix cancer cell line known to be amenable to RNAi [21]. A variable degree of telomerase activity inhibition as a function of the different oligomers (ranging from about 10 to 85% with respect to control siRNA-treated cells) was observed. When prostate cancer cell lines were transfected with hTERT-specific siRNAs, telomerase inhibition ranged from 3.5 ± 12.3 to 81.4 ± 0.8% in PC-3 cells and from 0 to 81.9 ± 1.9% in DU145 cells with respect to control siRNA-treated cells (Fig. 2a and b). By contrast, the transfection agent and control siRNA failed to affect telomerase activity in both prostate cancer cell lines.

(a)

siRNA number	Target sequence 5'-AA(N_{19})-3'	Position
2	aaggagcugguggcccgagug	285
5	aacacggugaccgacgcacug	426
6	aaggcgucugggaugcgaacg	635
8	aaccauagcgucagggaggcc	663
10	aagaggcccaggcguggcgcu	759
13	aagcacuuccucuacuccuca	1038
16	aaccacgcgcagugccccuac	1248
22	aagaaguucaucucccugggg	1527
27	aaguuccugcacuggcugaug	1680
31	aagagugucuggagcaaguug	1785
35	aagagggugcagcugcgggag	1833
41	aaaagagggccgagcgucuca	1998
56	aagagccacgucucuaccuug	2331
63	aagaagccaccucuuuggagg	892

(b)

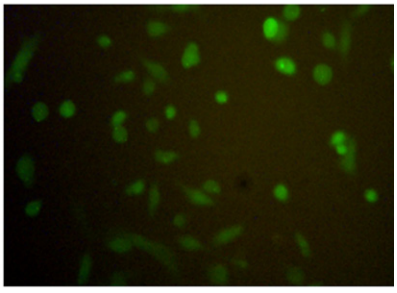

Fig. 1 – Small interfering RNAs targeting hTERT. (a) Position of siRNAs target sequences (5′-AA(N_{19})-3′) within hTERT mRNA (GeneBank accession no. AF015950). (b) Representative photograph showing the uptake and cellular distribution pattern of FAM-conjugated siRNAs in prostate cancer PC-3 cells, as detected by fluorescence microscopy.

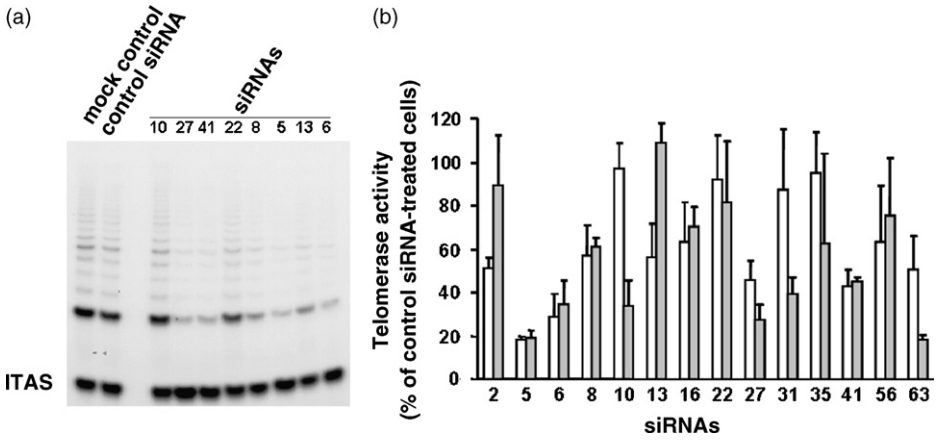

Fig. 2 – Inhibition of telomerase activity in prostate cancer cells exposed to hTERT-specific siRNAs. (a) Representative telomeric repeats amplification protocol (TRAP) experiment performed on protein extracts collected 2 days after a 4 h exposure of PC-3 cells either to Lipofectamine-2000™ (mock control) or to 125 nM siRNAs. The location of the internal amplification standard (ITAS) is reported. (b) Quantification of telomerase activity (as a percentage of the activity observed in control-siRNA-treated cells) of PC-3 (white column) and DU145 (grey column) cells exposed to 125 nM of siRNAs. Data represent the mean values ± S.D. of at least three independent experiments.

3.2. siRNAs markedly reduce hTERT mRNA and protein expression levels in prostate cancer cells

The effects consequent to cell transfection with siRNAs targeting hTERT were assessed using siRNA5 and siRNA41, which were able to inhibit the enzyme's catalytic activity by ~80 and ~55%, respectively, in PC-3 and DU145 cell lines (Fig. 2b). Results of RT-PCR experiments on total RNA obtained from both cell lines 2 days after a 4-h transfection with 125 nM of each siRNA showed that hTERT mRNA expression levels were down-regulated in both cell lines, although the inhibition was more pronounced in PC-3 than in DU145 cells (Fig. 3a). To quantify the extent of siRNA-mediated hTERT down-regulation, real-time RT-PCR was carried out in these cells. Results obtained 2 days after transfection confirmed that PC-3 cells exposed to siRNA5 and siRNA41 had reduced hTERT mRNA expression levels with respect to cells transfected with control siRNA: 45 ± 5% ($P < 0.01$) and 30 ± 7% ($P < 0.001$), respectively (Fig. 3b). Although to a lesser degree, a reduction of hTERT mRNA expression levels was also detected in DU145 cells: 70 ± 3% ($P < 0.01$) and 65 ± 6% ($P < 0.01$) with respect to control siRNA-trasfected cells in siRNA5 and siRNA41-treated cells, respectively (Fig. 3b).

Results of Western blotting experiments (Fig. 3c) showed that 2 days after transfection of PC-3 with siRNA5 and siRNA41, hTERT protein expression was reduced by about 60% with respect to that observed in cells transfected with control siRNA. Such an inhibition remained stable at day 3 and was still present, although to a lesser degree, at day 4 (Fig. 3d). As far as DU145 are concerned, a marked hTERT protein inhibition was appreciable 2 and 3 days after transfection with siRNA5 and only 2 days after exposure to siRNA41 (Fig. 3d).

3.3. siRNA5 and siRNA41 impair the proliferative potential of prostate cancer cells

A 4-h exposure to 125 nM siRNA5 or siRNA41 resulted in significant cell growth inhibition with respect to control siRNA in both cell lines, which was already appreciable 3 days after treatment removal and still present after 6 days of recovery (Fig. 4a). Specifically, in PC-3 cells, the percentage inhibition ranged from 36.4 to 40.3% and from 36.4 to 58.1% at the two time points after exposure to siRNA5 and siRNA41, respectively (Fig. 4a), whereas in DU145 cells, growth inhibition values ranged from 55.2 to 60.0% and from 53.2 to 57.4%, respectively (Fig. 4a).

To verify whether cell growth delay was due to an enhancement of the spontaneous apoptotic rate in siRNA-transfected cells, we checked by flow cytometry for the presence of the sub-G_1 peak, which is suggestive of apoptosis [22]. No sub-G_1 peak was observed in DU145 cells exposed to control siRNA, whereas peaks accounting only for 7.2 ± 2.2 and 6.2 ± 2.0% of the overall cell population were present in DU145 cells 3 days after the transfection with siRNA5 or siRNA41, respectively (Fig. 4b). A negligible apoptotic response was observed in hTERT-specific siRNA-transfected PC-3 cells (data not shown).

3.4. siRNA-mediated biological effects do not arise as a consequence of telomere shortening or loss of telomeric 3′ overhangs

These short-term effects on cell proliferation were not paralleled by a significant telomere shortening, as assessed by TRF analysis (Fig. 5a). Indeed, Southern blot hybridization carried out 3 days after the exposure of PC-3 and DU145 cells to siRNAs showed that TRF length ranged from approximately 3.0 to 8.0 kb, with a mean value of 5.0 (PC-3), and from 2.0 to 5.0 kb, with a mean value of 3.8 (DU145), in both control and hTERT-specific siRNA-treated cells. To get insight into the mechanism of such a rapid cell growth inhibition, we checked whether telomere uncapping occurred. The in solution hybridization assay failed to reveal a marked impairment of telomeric 3′ overhang in both cell lines after transfection with siRNAs (Fig. 5b). In addition, microscopy analysis of Hematoxylin/

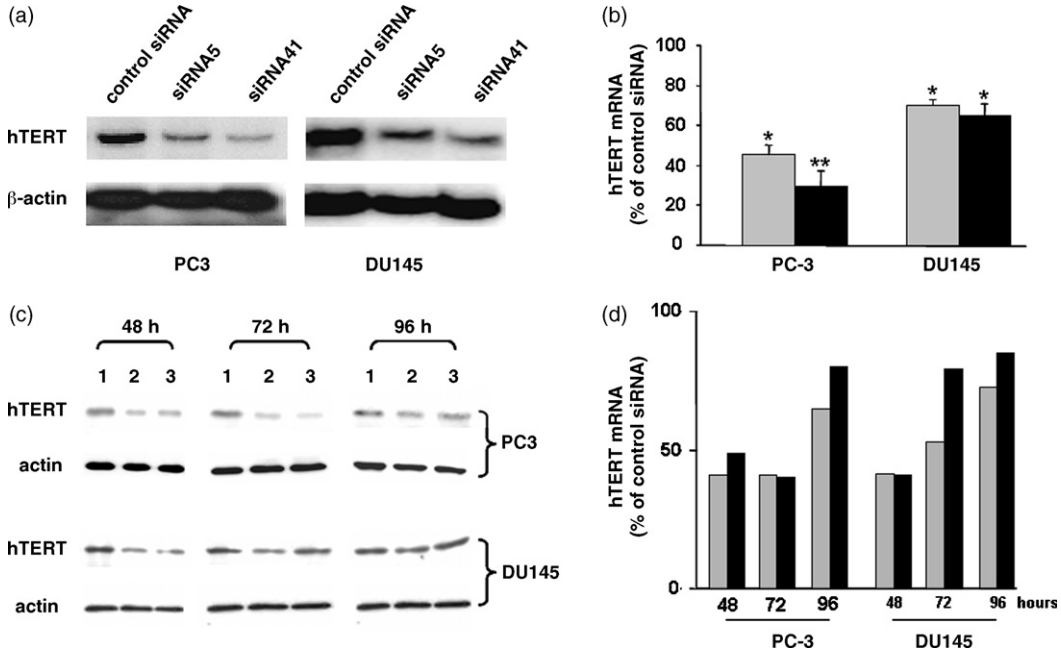

Fig. 3 – Down-regulation of hTERT mRNA and protein expression levels in prostate cancer cells exposed to siRNA5 and siRNA41. (a) Representative RT-PCR experiment showing the modulation of hTERT mRNA expression, performed on RNA collected 2 days after a 4-h exposure of PC-3 and DU145 cells to 125 nM control siRNA, siRNA5 or siRNA41. A fragment corresponding to β-actin was amplified as an internal control for PCR. (b) hTERT mRNA expression levels in PC-3 and DU145 cells exposed to siRNA5 (grey column) or siRNA41 (black column) quantified by real time RT-PCR and expressed as a percentage of control siRNA-transfected cells. Relative quantification of hTERT mRNA expression levels was accomplished by the $2^{-\Delta\Delta C_T}$ method as described in Section 2. Data represent the mean values ± S.D. of at least three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$, Student's *t*-test. (c) Representative Western immunoblotting experiment showing the expression of hTERT protein at different time points after a 4-h exposure of PC-3 and DU145 cells to 125 nM control siRNA (lane 1), siRNA5 (lane 2) or siRNA41 (lane 3). (d) Quantification of hTERT protein expression levels (as a percentage of control siRNA-transfected cells) in PC-3 and DU145 cells exposed to siRNA5 (grey column) or siRNA41 (black column).

Eosin-stained cytospins obtained from both cell lines exposed to siRNA5 or siRNA41 showed the presence of anaphase bridges in a negligible percentage of cells, which was superimposable to that observed in control siRNA-treated cells (data not shown).

3.5. *siRNA5 and siRNA41 do not induce non-specific and off-target effects*

To confirm that the effects observed in PC-3 and DU145 prostate cancer cells were consequent to the interference with hTERT expression and not due to off-target effects, we investigated whether siRNA5 and siRNA41 interfered with the growth of hTERT-negative U2-OS osteosarcoma cell line. First we assessed the ability of such a cell line to undergo the activation of RNAi pathway. To this purpose a siRNA targeting a consensus sequence within the EGFP mRNA was designed and chemically synthesized (siRNA GFP). Fluorescence microscopy carried out 2 days after a 4-h transfection in the presence of both siRNA GFP (125 nM) and 1 μg of pEGFP-N1 vector showed a complete disappearance of green fluorescent signal in co-transfected U2-OS cells as compared to cells exposed to the EGFP-expressing vector alone. Conversely, EGFP expression was not significantly affected in cells co-transfected with 1 μg pEGFP-N1 vector and 125 nM siRNA5 (data not shown). Given the susceptibility to RNAi activation, U2-OS cells were exposed to 125 nM of siRNA5 or siRNA41. The transfection of such a telomerase-negative cell line with hTERT-specific siRNAs did not impair cell proliferation as showed by cell growth curves, which were superimposable to that of U2-OS cells transfected with control siRNA (Fig. 6a), during the whole time course of experiments.

Finally, to rule out the possibility that the immediate effects observed in prostate cancer cells were partially due to a siRNA-mediated activation of the interferon pathway, OAS-1 mRNA expression levels were evaluated. Results obtained by RT-PCR in DU145 cells 2 days after the exposure to increasing concentrations of siRNAs failed to show an appreciable modulation of the expression levels of such an interferon response gene, in hTERT-specific siRNA-treated cells compared to control siRNA transfectants (Fig. 6b). To further confirm the specificity of the effects induced by siRNA5 and siRNA41 on telomerase activity and cell proliferation, we used HCT 116 cell line which is known to have a defective interferon response pathway [23]. A 4-h exposure of HCT 116 cells to 125 nM of siRNA5 or siRNA41 induced a marked decrease of telomerase activity (around 80 and 70%, respectively) as assessed by TRAP assay 2 days after treatment removal (Fig. 6c). Moreover, an appreciable cell growth inhibition, similar to that obtained in PC-3 and DU145 prostate cancer cell lines, was observed starting from 1 day after treatment removal (Fig. 6d). Such an inhibitory effect was found

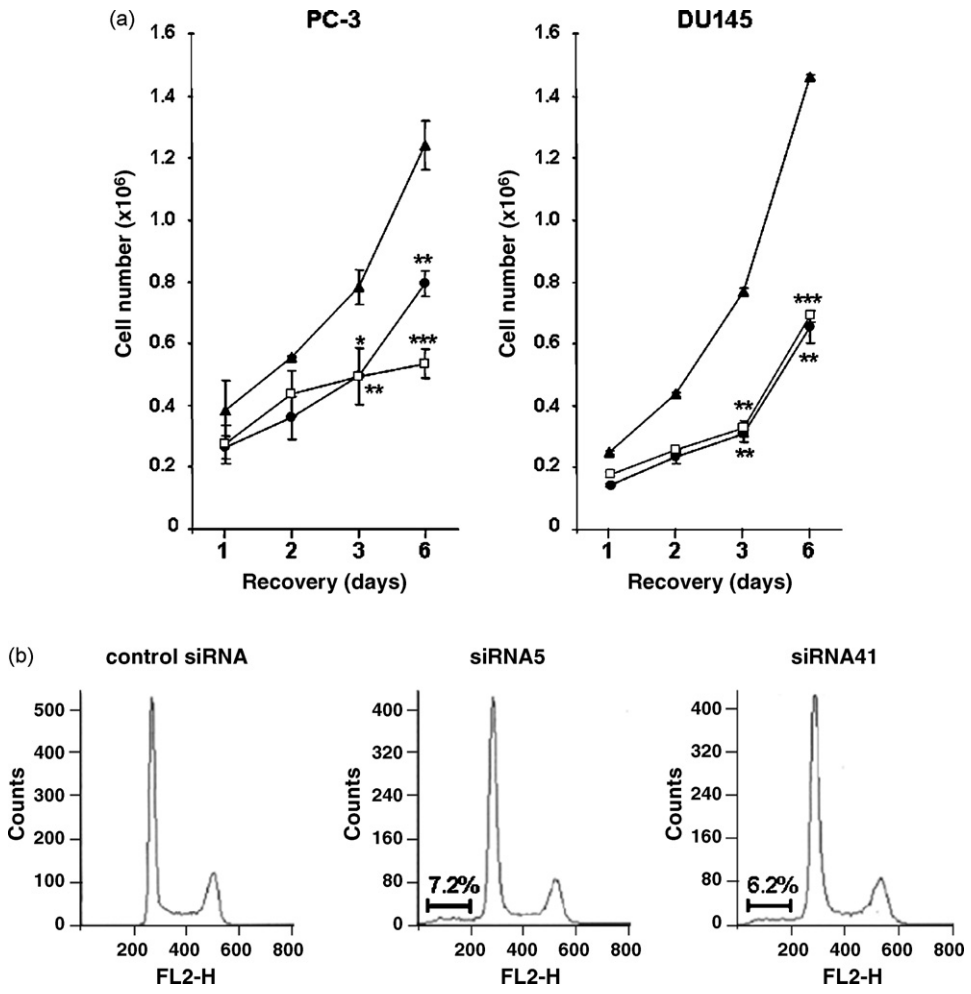

Fig. 4 – siRNA-mediated impairment of the proliferative potential of prostate cancer cells. (a) Proliferation of PC-3 and DU145 cells at different intervals of time after the exposure to 125 nM control siRNA (▲), siRNA5 (●) or siRNA41 (□). Data represent mean values ± S.D. of three independent experiments. (b) DNA content of DU145 cells exposed to control siRNA or hTERT-specific siRNAs as analyzed by fluorescence activated cell sorter at day 3. Bars indicate the cell population gated as sub-G_1 peak and the percentages of apoptotic cells are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Student's *t*-test.

to be related to the siRNA-mediated down-regulation of hTERT expression, since its forced overexpression in HCT 116 cells almost completely rescued cell proliferation. Specifically, the growth of HCT 116 double-transfectants, evaluated 3 days after a 4-h exposure to 125 nM siRNA5 or siRNA41 in the presence of 1 μg of pCI-neo-hTERT vector, was significantly ($P < 0.05$) less inhibited than that of cells transfected with siRNA5 or siRNA41 alone (Fig. 6e).

3.6. *hTERT-specific siRNA impairs the tumorigenicity of PC-3 cells in vivo*

The effect of siRNA-mediated inhibition of telomerase activity on tumorigenicity of PC-3 cells was studied in athymic nude mice following subcutaneous injection of untreated cells and cells transfected with control siRNA or hTERT-specific siRNAs. No significant difference in tumor take (Table 1) or growth pattern (Fig. 7) was evident between untreated cells and cells exposed to control siRNA. In contrast, siRNA5 negatively affected the in vivo take and growth of PC-3 cells. Specifically, at the different time points considered, the number of growing tumors originated from siRNA5-transfected cells was significantly lower than that observed for untreated cells and cells exposed to control siRNA (Table 1). Interestingly, the three

Table 1 – Effect of siRNA-mediated hTERT inhibition on tumorigenicity of PC-3 cells in nude mice

	No. of detectable tumors/no. of tumor cell injections			
	15[a]	20[a]	30[a]	50[a]
Untreated cells	10/10	10/10	10/10	–
Control siRNA	8/10	8/10	8/10	–
siRNA5	3/10	0/10[b]	2/10[c]	5/10
siRNA41	4/10	7/10	7/10	5/8

[a] Days.
[b] $P = 0.007$ vs. control siRNA; Fisher's exact test.
[c] $P = 0.025$ vs. control siRNA; Fisher's exact test.

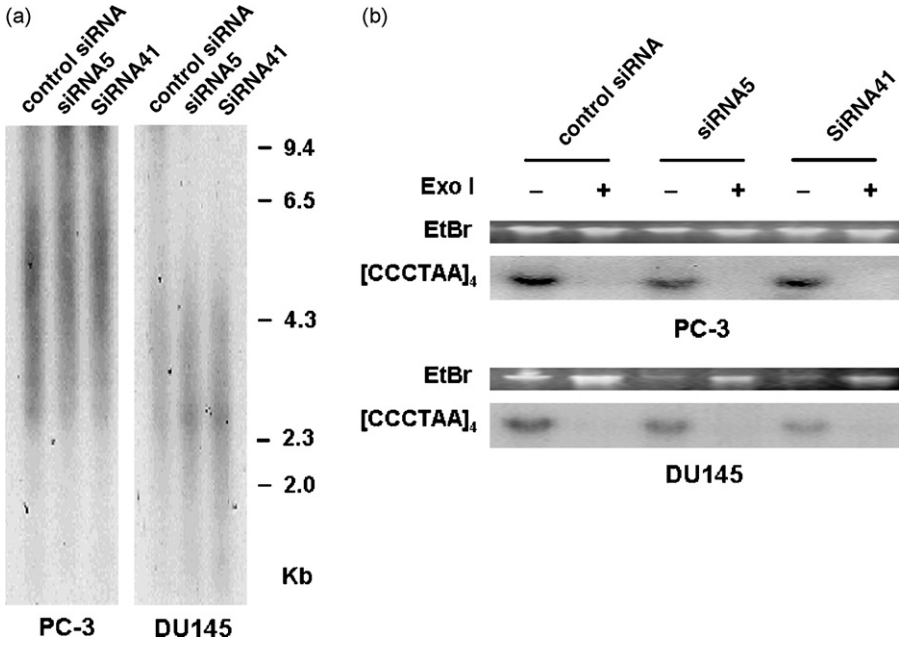

Fig. 5 – Telomere length and telomeric 3′-overhangs in prostate cancer cells exposed to siRNAs. (a) Southern blot analysis of telomeric restriction fragment (TRF) carried out 3 days after the exposure of PC-3 and DU145 cells to 125 nM control siRNA, siRNA5 or siRNA41. (b) Representative in solution hybridization assay showing the telomeric 3′ overhang status of PC-3 and DU145 cells, performed on native DNA collected from cells exposed to 125 nM control siRNA, siRNA5 or siRNA41. Exo I: DNA undigested (−) or digested (+) with single strand specific Exonuclease I; EtBr: signal of ethidium bromide stained DNA; $[CCCTAA]_4$: signal of DNA hybridized with ^{32}P-labeled telomeric 3′ overhang specific probe.

measurable tumors present at the day 6 in the group of animals inoculated with siRNA5-transfected cells progressively decreased in size with time, and no palpable tumor nodule was detectable at the day 20. Transfection with siRNA5 substantially delayed the regrowth of tumors (Fig. 7 and Table 1), and only five tumors derived from siRNA5-transfected cells were appreciable at the end of the experiment. In addition, a modest and temporary effect was exerted by siRNA41 on tumor take and growth pattern of PC-3 cells. Specifically, a 50% reduction in the number of growing tumors originated from siRNA41-transfected cells with respect to that observed for cells exposed to control siRNA was appreciable only until day 15 (Table 1). Similarly, a decrease in the mean size was appreciable only until day 20 for tumors originated from siRNA41-transfected cells compared to that observed for cells exposed to control siRNA (Fig. 7).

4. Discussion

The use of siRNAs to efficiently induce sequence-specific gene silencing in mammalian cells has opened new opportunities in the field of oncology for the validation of new therapeutic targets and the development of innovative anticancer therapies based on the interference with the expression of specific cancer-related genes [16]. Despite the assumed unique potential of RNAi, limitations in the use of such an approach have been described. In fact, when targeting endogenous lamin A/C mRNA in human HeLa or mouse SW3T3 cells, cell-type-dependent global effects and cell-type-independent positional effects were found [21], being HeLa cells about two-fold more responsive to siRNAs than SW3T3 cells, in spite of a very similar pattern of positional variation of lamin A/C silencing in both cell lines [21]. In this study fourteen chemically synthesized siRNAs targeting different consensus sequences within hTERT mRNA were used. Our results showed that siRNAs were generally able to induce inhibition of telomerase activity in PC-3 and DU145 prostate cancer cells, even though at a different extent as a function of the cell line and the oligomer used. This finding indicated that siRNAs were effective in eliciting the RNAi pathway in both prostate cancer cells even though a strong positional effect, due to a different accessibility of their target sites likely occurred. In fact, it has been shown that accessible siRNA target sites may be rare in some human mRNAs [24], probably as a consequence of local secondary structures, which may limit target accessibility [25–27].

Due to the positional effect observed with siRNAs targeting hTERT, we focused on siRNA5 and siRNA41, which were able to induce a marked and comparable inhibition of the enzyme's catalytic activity in both PC-3 and DU145 cells, to assess the consequence of telomerase down-regulation on cell proliferative potential. In prostate cancer cells transfected with siRNA5 and siRNA41, the decline of telomerase activity was paralleled by a significant reduction of hTERT mRNA and protein expression, although the inhibition extents were lower in DU145 than in PC-3 cells. Such a finding could be tentatively explained either by a different timing of siRNA-induced effects in DU145 cells with respect to PC-3 cells or as a consequence of a lower susceptibility of DU145 cells to undergo the activation

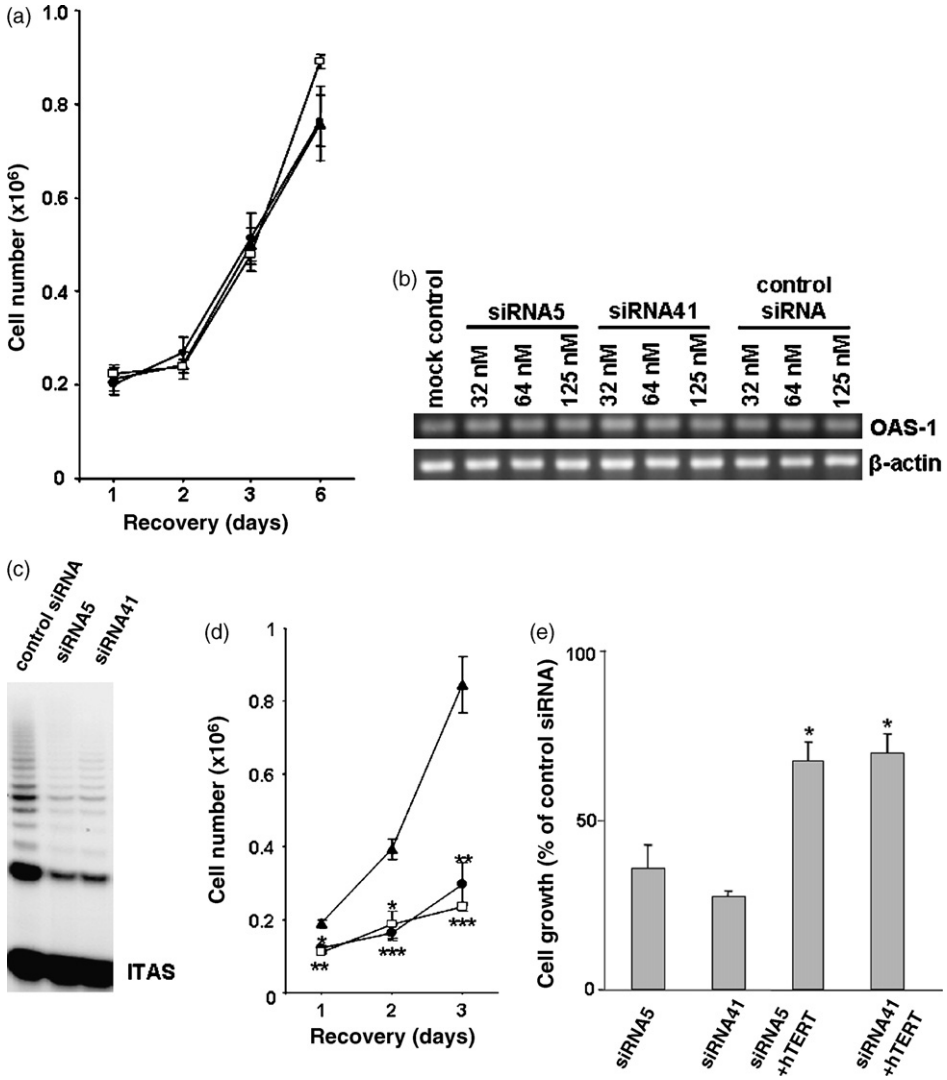

Fig. 6 – Analysis of off-target and non-specific effects in prostate cancer cells exposed to siRNAs. (a) Proliferation of U2-OS cells after the exposure either to 125 nM control siRNA (▲), siRNA5 (●) or siRNA41 (□). Data represent mean values ± S.D. of three independent experiments. (b) Representative RT-PCR experiment showing OAS-1 mRNA expression levels, performed on RNA collected 2 days after a 4-h exposure of DU145 cells to different doses of siRNAs. A fragment corresponding to β-actin was amplified as an internal control for PCR. (c) Representative TRAP assay performed on protein extracts collected 2 days after a 4-h exposure of HCT 116 cells to 125 nM control siRNA, siRNA5 or siRNA41. The location of the internal amplification standard (ITAS) is reported. (d) Proliferation of HCT 116 cells at different intervals of time after the exposure to 125 nM control siRNA (▲), siRNA5 (●) or siRNA41 (□). Data represent mean values ± S.D. of three independent experiments. (e) Proliferation of transfectants evaluated 3 days after a 4-h exposure of HCT 116 cells to 125 nM siRNA5 or siRNA41 alone or in the presence of 1 μg of an hTERT-expressing vector (pCI-neo-hTERT). Data are expressed as percentage of cell number with respect to cells exposed to control siRNA and represent mean values ± S.D. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.02$; $^{***}P < 0.01$. Student's *t*-test.

of RNAi pathway as compared to PC-3 cells, since it has been demonstrated that different mammalian cells may not be equally amenable to RNAi [21]. Despite the assumed differences in the susceptibility of PC-3 and DU145 to RNAi, we observed a retardation of cell growth after transfection with siRNA5 and siRNA41. Moreover, transfection of PC-3 cells with siRNA5 was able to significantly reduce their tumorigenic and growth potential after xenotransplantation in athymic nude mice, whereas a modest effect of siRNA41 transfection on in vivo take and growth of these cells was observed.

In accordance with our results, anti-proliferative effects of siRNAs targeting hTERT, delivered to cells by positively charged single-walled nanotubes, have been recently described in murine tumor cells and human HeLa cells both in vitro and in vivo [28]. In addition, the transient transfection of T24 bladder cancer cells and HepG2 and SMMC-7721 hepatocarcinoma cells with a DNA-based vector carrying short hairpin RNAs (shRNAs) against hTERT resulted in the suppression of cell growth in vitro and in vivo as the consequence of hTERT down-regulation, inhibition of telo-

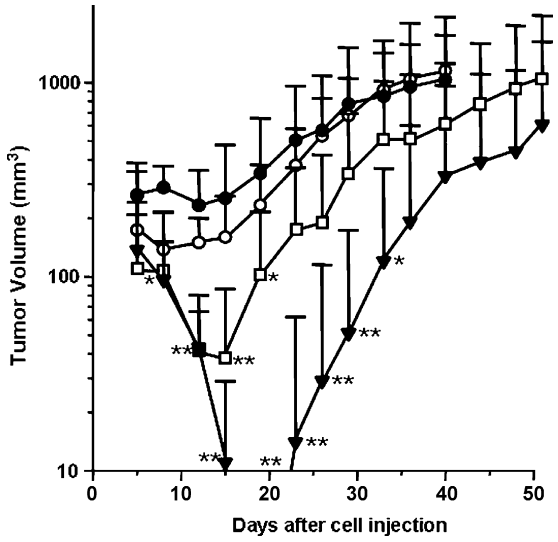

Fig. 7 – Effect of siRNA-mediated hTERT inhibition on the tumorigenic potential of human prostate cancer cells. Exponentially growing PC-3 cells, untreated (○) or exposed to 125 nM control siRNA (●), siRNA5 (▼) or siRNA41 (□), were injected subcutaneously into both flanks of athymic nude mice (10^7 cells/flank). Tumor volumes were calculated as described in Section 2. Data are expressed as mean tumor volumes ± S.D. $^{*}P < 0.05$; $^{**}P < 0.01$; Student's *t*-test.

merase activity and the concomitant reduction of c-myc expression levels [29,30].

Our results also showed that cell growth impairment occurred in the absence of any siRNA-mediated effect on telomere length or status. Specifically, no TRF shortening, telomeric 3′ overhang impairment and appearance of anaphase bridges was observed in either cell line. Since it has been reported that siRNAs can cause off-target effects or be potent inducers of stress-response pathway [31–37], which results in a global up-regulation of interferon-stimulated genes, we performed an extensive analysis to rule out the possibility that the antiproliferative effect observed in prostate cancer cells was a consequence of siRNA-mediated side effects. Exposure of the hTERT-negative U2-OS osteosarcoma cells to siRNA5 and siRNA41 excluded the presence of off-target effects, since the treatment failed to affect cell growth in spite of the ability of osteogenic sarcoma cells to activate RNAi pathway. In addition, siRNA5 and siRNA41 were able to induce a significant retardation of cell growth of HCT 116 colon cancer cells, which are characterized by a deregulation of the interferon response pathway, and such a phenotype was also almost completely rescued in HCT 116 cells overexpressing hTERT target. Finally, the levels of OAS-1 mRNA (an effector of the interferon-mediated stress response [38,39]) were not significantly up-regulated in DU145 cells exposed to siRNAs. Altogether, these data corroborate the idea that siRNA-mediated cell growth impairment observed in PC-3 and DU145 cells is not ascribable to a non-specific activation of the RNAi machinery but is rather a consequence of specific hTERT down-regulation.

It should be also taken into account that siRNAs may induce complex signaling responses in target cells beyond the selective silencing of specific genes [40,41]. In this context, Li et al. [39] showed that a shRNA, expressed from a lentiviral vector and targeting the sequence encompassing the 11-nt template region of hTR, induced a rapid inhibition of the growth of p53-wild-type and p53-null HCT 116 and LOX melanoma cells, independently of p53 status or telomere length within a few days [39]. Such a rapid cellular response did not occur as a consequence of either telomere attrition or telomere uncapping, since a bulk telomere shortening and the increase of telomerically located DNA damage foci (an experimental evidence of telomere uncapping) were not observed [39]. Conversely, the inhibition of telomerase activity in HCT 116 cells by the shRNA-expressing vector induced a change in the expression of genes which were distinct from those involved in the DNA damage and telomere-uncapping response pathways induced in the same cell line by a telomere-uncapping mutant template telomerase RNA [39,42].

The impairment of cancer cell growth by specific hTERT targeting can be sustained either by telomere shortening, as a result of prolonged inhibition of telomere lengthening activity of telomerase, or by loss of telomere lengthening-independent functions of hTERT [43,44]. Our findings are in trend with data previously obtained in our and other laboratories, reporting that the down-regulation of hTERT expression, accomplished through the use of antisense oligonucleotides or ribozymes, quickly impaired the cell growth and induced a variable degree of programmed cell death within a few days in several human tumor models [13]. Although to a modest extent, in this study we observed the presence of a sub-G_1 peak on DNA plots of prostate cancer cells exposed to siRNA5 or siRNA41, which was suggestive of apoptosis induction. The low efficiency with which siRNA5 and siRNA41 induced the down-regulation of hTERT in DU145 cells and the characteristic slow cell death and delayed DNA fragmentation of PC-3 cells [45] would possibly account for the scanty induction of apoptosis in these cell lines. However, in keeping with our findings it has been recently demonstrated that normal human fibroblasts, expressing hTERT during the S phase of the cell cycle, were characterized by a marked cell growth retardation but not by a significant induction of apoptosis when infected by a viral vector encoding for a hTERT-specific shRNA [46]. It should also be taken into account that the global effects we observed in prostate cancer cells occurred within a few days after a single administration of siRNA5 or siRNA41, exogenously delivered into cells as single agents. Conversely, in a recent study it was shown that a long-term suppression of hTERT by an siRNA-expressing retroviral vector affected the proliferative and tumorigenic potential of HeLa cells as a consequence of telomerase activity inhibition, telomere shortening and loss of telomeric 3′ overhangs [47]. In keeping with this evidence, it was also observed that HT29 colorectal adenocarcinoma cells stably expressing hTERT-specific shRNA displayed reduced telomere lengths and stopped proliferation [48]. In addition, HeLa clones stably transfected with an expression vector encoding a shRNA against hTERT showed extremely reduced hTERT mRNA associated with short telomeres, inhibition of cell growth and induction of senescence and apoptosis [49]. These observations are not in contrast with our data, since the

discrepancy in the timing of cell growth inhibitory effects and their association with telomere shortening could reside in the different methodological approach used. In fact, it has been hypothesized that the long-term selection of stable shRNA-expressing clones could mask the biological effects observable immediately after the exposure of cells to transiently delivered siRNAs [39].

In conclusion, our results revealed that hTERT-specific siRNAs can efficiently reduce the cell growth and tumorigenicity of prostate cancer cells by specifically inhibiting hTERT expression and telomerase activity, and corroborate earlier evidence suggesting that siRNA-mediated suppression of hTERT represents a powerful tool to counteract cancer cell growth. However, in our opinion the relative efficacy and specificity of a given siRNA need to be carefully established for each individual experimental model also through the use of stringent controls, and further studies are warranted to highlight the exact molecular mechanisms underlying RNAi-based post-transcriptional gene silencing.

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